

APPLICATION FOR UNITED STATES
LETTERS PATENT

for

METHODS OF ASSAYING RECEPTOR ACTIVITY AND CONSTRUCTS
USEFUL IN SUCH METHODS

by

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This application is a continuation of application Serial No. 09/469,554 filed December 22, 1999, entitled Inhibition of β -arrestin Mediated Effects Prolongs and Potentiates Opioid Receptor-Mediated Analgesia; which is a continuation in part of Serial No. 09/233,530, filed January 20, 1999, entitled Methods of Assaying Receptor Activity and Constructs Useful in Such Methods, now U.S. Patent No. 6,110,693, issued August 29, 2000; which is a continuation of Serial No. 08/869,568, filed June 5, 1997, entitled Methods of Assaying Receptor Activity and Constructs Useful in Such Methods, now U.S. Patent No. 5,891,646, issued April 6, 1999.

FEDERALLY SPONSORED RESEARCH

This invention was made with Government support under National Institutes of Health Grant No. HL 03422-02, HL 16037, and NS 19576. The Government has certain rights to this invention.

FIELD OF THE INVENTION

This invention relates to methods of detecting G-protein coupled receptor (GPCR) activity in vivo and in vitro, and provides methods of assaying GPCR activity, and methods of screening for GPCR ligands, G protein-coupled receptor kinase (GRK) activity, and compounds that interact with components of the GPCR regulatory process.

This invention also provides constructs useful in such methods.

BACKGROUND OF THE INVENTION

The actions of many extracellular signals are mediated by the interaction of G-protein coupled receptors (GPCRs) and guanine nucleotide-binding regulatory proteins (G proteins). G protein-mediated signaling systems have been identified in many divergent organisms, such as mammals and yeast. GPCRs respond to, among other extracellular signals, neurotransmitters, hormones, odorants and light. GPCRs are similar and possess a number of highly conserved amino acids; the GPCRs are thought to

represent a large 'superfamily' of proteins. Individual GPCR types activate a particular signal transduction pathway; at least ten different signal transduction pathways are known to be activated via GPCRs. For example, the beta 2-adrenergic receptor (β AR) is a prototype mammalian GPCR. In response to agonist binding, β AR receptors activate a G protein (G_s) which in turn stimulates adenylate cyclase and cyclic adenosine monophosphate production in the cell.

It has been postulated that members of the GPCR superfamily desensitize via a common mechanism involving G protein-coupled receptor kinase (GRK) phosphorylation followed by arrestin binding. Gurevich et al., *J. Biol. Chem.* 270:720 (1995); Ferguson et al., *Can. J. Physiol. Pharmacol.* 74:1095 (1996). However, the localization and the source of the pool of arrestin molecules targeted to receptors in response to agonist activation was unknown. Moreover, except for a limited number of receptors, a common role for β -arrestin in GPCR desensitization had not been established. The role of β -arrestins in GPCR signal transduction was postulated primarily due to the biochemical observations.

Many available therapeutic drugs in use today target GPCRs, as they mediate vital physiological responses, including vasodilation, heart rate, bronchodilation, endocrine secretion, and gut peristalsis. See, eg., Lefkowitz et al., *Ann. Rev. Biochem.* 52:159 (1983). GPCRs include the adrenergic receptors (alpha and beta); ligands to beta ARs are used in the treatment of anaphylaxis, shock, hypertension, hypotension, asthma and other conditions. Additionally, spontaneous activation of GPCRs occurs, where a GPCR cellular response is generated in the absence of a ligand. Increased spontaneous activity can be decreased by antagonists of the GPCR (a process known as inverse agonism); such methods are therapeutically important where diseases cause an increase in spontaneous GPCR activity.

Efforts such as the Human Genome Project are identifying new GPCRs ('orphan' receptors) whose physiological roles and ligands are unknown. It is estimated that several thousand GPCRs exist in the human genome. With only about 10% of the human

genome sequenced, 250 GPCRs have been identified; fewer than 150 have been associated with ligands.

SUMMARY OF THE INVENTION

5 A first aspect of the present invention is a conjugate of a β -arrestin protein and a detectable molecule. The detectable molecule may be an optically detectable molecule, such as Green Fluorescent Protein.

10 A further aspect of the present invention is a nucleic acid construct comprising an expression cassette. The construct includes, in the 5' to 3' direction, a promoter and a nucleic acid segment operatively associated with the promoter, and the nucleic acid segment encodes a β -arrestin protein and detectable molecule. The detectable molecule may be an optically detectable molecule such as Green Fluorescent Protein.

15 A further aspect of the present invention is a host cell containing a nucleic acid molecule which includes, a promoter operable in the host cell and a nucleic acid sequence encoding a β -arrestin protein and a detectable molecule. The detectable molecule may be an optically detectable molecule such as Green Fluorescent Protein. The cell may be a mammalian, bacterial, yeast, fungal, plant or animal cell, and may be deposited on a substrate.

20 A further aspect of the present invention is a method of assessing G protein coupled receptor (GPCR) pathway activity under test conditions, by providing a test cell that expresses a GPCR and that contains a conjugate of a β -arrestin protein and a visually detectable molecule; exposing the test cell to a known GPCR agonist under test conditions; and then detecting translocation of the detectable molecule from the cytosol of the test cell to the membrane edge of the test cell. Translocation of the detectable molecule in the test cell indicates activation of the GPCR pathway. Exemplary test conditions include the presence in the test cell of a test kinase and/or a test G-protein, or exposure of the test cell to a test ligand, or co-expression in the test cell of a second receptor.

A further aspect of the present invention is a method for screening a β -arrestin protein (or fragment of a β -arrestin protein) for the ability to bind to a phosphorylated GPCR. A cell is provided that expresses a GPCR and contains a conjugate of a test β -arrestin protein and a visually detectable molecule. The cell is exposed to a known
5 GPCR agonist and then translocation of the detectable molecule from the cell cytosol to the cell edge is detected. Translocation of the detectable molecule indicates that the β -arrestin molecule can bind to phosphorylated GPCR in the test cell.

A further aspect of the present invention is a method to screen a test compound for G protein coupled receptor (GPCR) agonist activity. A test cell is provided that
10 expresses a GPCR and contains a conjugate of a β -arrestin protein and a visually detectable molecule. The cell is exposed to a test compound, and translocation of the detectable molecule from the cell cytosol to the membrane edge is detected. Movement of the detectable molecule to the membrane edge after exposure of the cell to the test compound indicates GPCR agonist activity of the test compound. The test cell may
15 express a known GPCR or a variety of known GPCRs, or express an unknown GPCR or a variety of unknown GPCRS. The GPCR may be, for example, an odorant GPCR or a β -adrenergic GPCR. The test cell may be a mammalian, bacterial, yeast, fungal, plant or animal cell.

A further aspect of the present invention is a method of screening a sample
20 solution for the presence of an agonist to a G protein coupled receptor (GPCR). A test cell is provided that expresses a GPCR and contains a conjugate of a β -arrestin protein and a visually detectable molecule. The test cell is exposed to a sample solution, and translocation of the detectable molecule from the cell cytosol to the membrane edge is assessed. Movement of the detectable molecule to the membrane edge after exposure to
25 the sample solution indicates the sample solution contains an agonist for a GPCR expressed in the cell.

A further aspect of the present invention is a method of screening a test compound for G protein coupled receptor (GPCR) antagonist activity. A cell is provided that

expresses a GPCR and contains a conjugate of a β -arrestin protein and a visually detectable molecule. The cell is exposed to a test compound and to a GPCR agonist, and translocation of the detectable molecule from the cell cytosol to the membrane edge is detected. When exposure to the agonist occurs at the same time as or subsequent to exposure to the test compound, movement of the detectable molecule from the cytosol to the membrane edge after exposure to the test compound indicates that the test compound is not a GPCR antagonist.

A further aspect of the present invention is a method of screening a test compound for G protein coupled receptor (GPCR) antagonist activity. A test cell is provided that expresses a GPCR and contains a conjugate of a β -arrestin protein and a visually detectable molecule. The cell is exposed to a GPCR agonist so that translocation of the detectable molecule from the cytosol of the cell to the membrane edge of the cell occurs, and the cell is then exposed to a test compound. Where exposure to the agonist occurs prior to exposure to the test compound, movement of the detectable molecule from the membrane edge of the cell to the cytosol after exposure of the cell to the test compound indicates that the test compound has GPCR antagonist activity.

A further aspect of the present invention is a method of screening a cell for the presence of a G protein coupled receptor (GPCR). A test cell is provided that contains a conjugate of a β -arrestin protein and a visually detectable molecule. The test cell is exposed to a solution containing a GPCR agonist. Any translocation of the detectable molecule from the cytosol to the membrane edge is detected; movement of the detectable molecule from the cytosol to the membrane edge after exposure of the test cell to GPCR agonist indicates that the test cell contains a GPCR.

A further aspect of the present invention is a method of screening a plurality of cells for those cells which contain a G protein coupled receptor (GPCR). A plurality of test cells containing a conjugate of a β -arrestin protein and a visually detectable molecule are provided, and the test cells are exposed to a known GPCR agonist. Cells in which the detectable molecule is translocated from the cytosol to the membrane edge are identified

or detected. Movement of the detectable molecule to the membrane edge after exposure to a GPCR agonist indicates that the cell contains a GPCR responsive to that GPCR agonist. The plurality of test cells may be contained in a tissue, an organ, or an intact animal.

5 A further aspect of the present invention is a substrate having deposited thereon a plurality of cells that express a GPCR and that contain a conjugate of a β -arrestin protein and a detectable molecule. Such substrates may be made of glass, plastic, ceramic, semiconductor, silica, fiber optic, diamond, biocompatible monomer, or biocompatible polymer materials.

10 A further aspect of the present invention is an apparatus for determining GPCR activity in a test cell. The apparatus includes means for measuring indicia of the intracellular distribution of a detectable molecule, and a computer program product that includes a computer readable storage medium having computer-readable program code means embodied in the medium. The computer-readable program code means includes
15 computer-readable program code means for determining whether the indicia of the distribution of the detectable molecule in a test cell indicates concentration of the detectable molecule at the cell membrane, based on comparison to the measured indicia of the intracellular distribution of a detectable molecule in a control cell. The indicia of the intracellular distribution of the detectable molecule may be optical indicia, and the
20 measuring means may be means for measuring fluorescent intensity. The molecule to be detected may be one that is fluorescently detectable, and the step of measuring the indicia of the intracellular distribution of the detectable molecule may include measurement of fluorescence signals from test and control cells.

25 A further aspect of the present invention is an apparatus for determining GPCR activity in a test cell. The apparatus includes means for measuring indicia of the intracellular distribution of a detectable molecule in at least one test cell at multiple time points, and a computer program product. The computer program product includes a computer readable storage medium having computer-readable program code means

embodied in said medium. The computer-readable program code means includes computer-readable program code means for determining whether the indicia of the distribution of the detectable molecule in the test cell at multiple time points indicates translocation of the detectable molecule to the cell membrane.

5 A further aspect of the present invention is an apparatus for determining GPCR activity in a test cell, which includes means for measuring indicia of the intracellular distribution of a detectable molecule in at least one test cell, and a computer program product. The computer program product includes a computer readable storage medium having computer-readable program code means embodied therein and including
10 computer-readable program code means for determining whether the indicia of the distribution of the detectable molecule in the test cell indicates concentration of the detectable molecule at the cell membrane, based on comparison to pre-established criteria.

Pain perception (nociception) is mediated by a cascade of events from the point of
15 the stimulus to integrative circuits in the brain. Nociception involves signals that are mediated by several classes of receptors and signal transduction mechanisms such as GPCRs for substance P, opioid peptides, etc. and ion channels such as NMDA receptors. Antinociception has been known for more than 1000 years to be induced by the alkaloid compound, morphine, which functions as an agonist at the μ -opioid receptor. The activity
20 of agonists for signaling through GPCRs is usually limited by cellular mechanisms that dampen the signal of the agonist, a process referred to as desensitization. These mechanisms include phosphorylation of agonist-activated receptors by specific receptor kinases called GRKs followed by the interaction of the phosphorylated GPCR with any of the members of the arrestin family of proteins. Morphine-mediated antinociception is
25 known to wane with time, however the contribution of the desensitization is controversial and for all practical purposes is unknown. With the β arrestin knockout mice disclosed herein, it is shown that interfering with (eliminating) one of the key protein components

of the desensitization mechanism greatly enhances the potency and efficacy of the antinociceptive properties of morphine.

Accordingly, an additional aspect of the present invention is a knockout mouse useful for testing the efficacy of potential analgesic agents, the cells of said mouse containing at least one inactive endogenous β arrestin gene (preferably the β arrestin-2 gene), the mouse exhibiting a phenotype of decreased sensitivity to pain after administration of a μ opioid receptor agonist such as morphine as compared to the corresponding wild type mouse. The mouse may be heterozygous or homozygous for the inactive endogenous β arrestin gene. The mouse is useful for evaluating potential analgesic drugs, and particularly for evaluating the contribution of the desensitization mechanisms to the antinociceptive effects of endogenous opioids.

BRIEF DESCRIPTION OF THE DRAWINGS

FIG. 1 is a linear model of the β -arrestin2/S65T-Green Fluorescent Protein (GFP) conjugate.

FIG. 2A provides the results of a Western Blot of homogenates of HEK-293 cells expressing the β arr2-GFP conjugate as well as endogenous β -arrestin2. β arr2 indicates endogenous cellular β -arrestin2; β arr2-GFP indicates β arrestin2-GFP conjugate; approximate molecular weights are indicated to the right of the gel. Land 1 was treated with anti- β arrestin antibody; Land 2 with anti-GFP antibody.

FIG. 2B shows the sequestration of β 2AR in COS cells with and without overexpressed β -arrestin2 (left two bars) and with and without overexpressed β arr2-GFP (right two bars). Wild type β -arrestin2 and β arr2-GFP enhanced β 2AR sequestration equally well above control levels, producing a 2.5 and 2.4 fold increase, respectively.

FIG. 3A: Confocal microscopy photomicrographs show β arr2-GFP translocation from cytosol (panel 1 at left) to membrane (panel 2 at right) in HEK-293 cells containing the β 2AR, due to the addition of the β AR2 agonist isoproterenol. Bar=10 microns.

FIG. 3B: Confocal microscopy photomicrographs show β arr2-GFP translocation

from cytosol (panel 1 at left) to membrane (panel 2 at right) in COS cells containing the β 2AR, and due to addition of the β AR2 agonist isoproterenol. Bar=10 microns.

FIG. 4 depicts a HEK-293 cell containing 12CA5(HA) tagged β 2AR (confocal microscopic photographs). FIG. 4A shows a cell after reorganization of β 2AR into plasma membrane clusters. FIG. 4B provides three pictures of the same cell at 0, 3, and 10 minutes (left to right) after the addition of agonist. Redistribution of β arr2-GFP to the cell membrane is shown by the enhancement of membrane fluorescence with a concomitant loss of cytosolic fluorescence. Arrows indicate areas of co-localization; bar=10 microns.

FIG. 5 shows the influence of overexpressed GRK on the redistribution of β arr2-GFP in HEK-293 cells expressing the Y326A phosphorylation-impaired β 2AR. Cells without (Row A) and with (Row B) overexpressed GRKs were exposed to agonist, and the real-time redistribution of β arr2-GFP was observed. β arr2-GFP translocation in cells containing overexpressed GRK (Row B) was more robust, indicating an increased affinity of β arr2-GFP for receptor. Bar=10 microns.

FIG. 6A depicts the agonist-induced time dependent translocation of β arr2-GFP to beta2 adrenergic receptors in a representational HEK-293 cell.

FIG. 6B graphs the time course of agonist-induced translocation of β arr2-GFP to beta2 adrenergic receptors in HEK-293 cells; this graph is quantitative and is based on the responses of a plurality of cells.

FIG. 6C is depicts the agonist-induced translocation of β arr2-GFP to beta2 adrenergic receptors in representational HEK-293 cells, at varying doses of agonist.

FIG. 6D graphs the dose dependent agonist-induced translocation of β arr2-GFP to beta2 adrenergic receptors in HEK-293 cells; this graph is quantitative and is based on the responses of a plurality of cells.

FIG. 6E evaluates the translocation of β arr2-GFP from the cell cytosol to the cell membrane, in response to exposure to receptor agonist (middle panel) and subsequent exposure to receptor antagonist (right panel).

FIG. 7 illustrates characteristics of the targeted disruption of the mouse β arrestin-2 (β arr2) gene.

FIG. 7A depicts schematic diagrams of β arr2 gene (top), targeting vector (middle) and the homologous recombinant gene (bottom) (7). The arrows indicate the translational start and stop sites. The black boxes indicate the exons. A 0.8 kb Bam HI-Hind III fragment was replaced with the pGK-neo cassette such that the entire exon 2, encoding amino acids 9-19, was deleted. Transcription of the neomycinresistant gene opposed that of the β arr2 gene. Both 5' and 3' external probes were used in genotype screening. Restriction enzyme sites are as follows: B, Bam HI; N, Nco 1; H, Hind III; R, Eco RI.

FIG. 7B illustrates southern blot analysis of genomic DNA from wild type (WT), heterozygous (+/-) and homozygous (-/-) mice. Tail DNA was digested with Bam HI and analyzed by Southern blotting with the 5' probe as shown in (A). A 3.5-kb fragment is indicative of the β arr2 knock-out (KO) allele and a 3-kb fragment is indicative of the wild-type allele.

FIG. 7C depicts protein immunoblot analysis of β arr2 expression in WT, β arr2 and β arr2-KO mice. Membranes were blotted for β arr1 (top) and β arr2 (bottom) protein expression. Each lane was loaded with 25 pLg protein derived from the same lysates of the indicated brain regions.

FIG. 8 illustrates enhanced and prolonged morphine-induced antinociception in β arr2-KO mice. Antinociceptive responses were measured as hot plate (56°C) response latency after morphine (10 mg/kg, s.c.) treatment. The "response" was defined by the animal either licking the fore- or hind-paws or flicking the hind-paws. In these studies, the most prominent response was fore-paw licking. To avoid tissue damage the animals were not exposed to the plate for more than 30 seconds. Data are reported as the percent of the maximal possible response time (30 seconds) which was determined from each individual mouse's basal response, the response after drug treatment, and the imposed maximum cutoff time with the following calculation (F. Porreca et al., *J Pharmacol Exp Ther* 230, 341 (1984); J. Belknap et al., *Physiol Behav* 46, 69 (1989). M. Gardmark et al.,

Pharmacol Toxicol 83, 252 (1998); G. Elmer et al., *Pain* 75, 129 (1998)): $100\% \times [(Drug \text{ response time} - Basal \text{ response time}) / (30 \text{ sec} - Basal \text{ response time})] = \% \text{ maximum possible effect (\% MPE)}$. WT (n = 6), heterozygotes (+/-, n = 5) and KO (n = 9) mice were analyzed together in the same experiment. The % MPE curves of the β arr2-KO and β arr2 +/- mice were significantly greater than the WT response curve ($P < 0.001$) as determined by two-way ANOVA.

FIG. 9 depicts greater dose-dependent antinociceptive responses to morphine in β arr2-KO mice. The degree of antinociception was determined by measuring latency of hot plate (56°C) responses (Fig. 2). Withdrawal latencies were measured 30 min. after a first dose of morphine (1 mg/kg, s.c.) at which point, animals were immediately injected with 4 mg/kg, s.c. morphine for a cumulative dose of 5 mg/kg. Antinociception was again assessed after 30 min. and mice were immediately injected with morphine (5 mg/kg, s.c.), to give a final cumulative dose of 10 mg/kg. Withdrawal latencies were again measured after 30 min. after which, mice were immediately injected with naloxone (2.5 mg/kg, s.c.). After 10 min., antinociception was assessed once more. WT (n = 7) and β arr2-KO (n = 6) mice were significantly different at each dose (* $P < 0.01$, ** $P < 0.001$; Student's *t*-test). Means \pm S.E.M. are shown. In an additional experiment, morphine (25 mg/kg, s.c.) induced the maximum imposed response (100%) in both genotypes. Thus, an approximate 2 fold shift in the apparent ED_{50} values was observed between genotypes [WT: 9.77 (8.0.8-11.81) mg/kg; KO: 5.98 (5.10-6.94) mg/kg (95% confidence intervals)].

FIG. 10 depicts increased hypothermic responses to morphine in β arr2-KO mice. Rectal body temperatures were measured with a digital thermometer (M. Adler et al., *Annu Rev Pharmacol Toxicol* 28, 429 (1988); F. Fumagalli et al., *J Neurosci* 18, 4861 (1998) (TH8, Physitemp, Clifton, New Jersey, USA). The probe was inserted into the rectum and maintained until the temperature reading stabilized. Basal body temperatures did not vary significantly between genotypes (WT: $36.4 \pm 0.1^\circ\text{C}$; KO: $36.8 \pm 0.1^\circ\text{C}$). VVT (n = 5) and KO (n = 5) animals were analyzed in parallel during the same

experiment. The curves are significantly different ($P < 0.001$) as determined by 2-way ANOVA.. Means \pm S.E.M. are shown.

FIG. 11 illustrates binding of [35 S]GTPYS to periaqueductal gray membranes from β arr2-KO and wild type (WT) mice. [35 S]GTPYS binding to isolated periaqueductal gray (PAG) membranes (prepared as described in conjunction with Table 1 below) was determined after 2 hour stimulation (30°C) with 50-10,000 nM of the mOR-selective agonist, [D-Ala2, MePhe4, Gly5-ol]enkephalin (DAMGO). PAG membranes (10 μ g protein per assay tube) were incubated in the presence of 10 μ M GDP and 50 pM [35 S]GTPYS (1250 Ci/mmol, NEN, Boston, MA). [35 S]GTPYS binding was measured as described (P. Portoghese, in Handbook of Experimental Pharmacology: Opioids 1, A. Herz, Ed. (Springer-Verlag, New York, 1993) p.p. 279-293. A. et al., *ibid*, p.p. 645-679). [35 S]GTPYS binding is expressed as percent increase in [35 S]GTPYS binding relative to binding in unstimulated samples. Data were analyzed by nonlinear regression using GraphPad Prism software and are presented as the mean + S.E.M of at least three experiments performed in triplicate wherein WT and β arr2-KO brain regions were assayed simultaneously. In the absence of agonist stimulation, basal [35 S]GTPYS binding was: WT: 440 ± 83 cpm and β arr2-KO: 527 ± 99 cpm.

Detailed Description of The Invention

Definitions

The term "arrestin " as used herein has its ordinary meaning in the art and is intended to encompass all types of arrestin, including but not limited to visual arrestin (sometimes referred to as Arrestin 1), β arrestin 1 (sometimes referred to as Arrestin 2), and β arrestin 2 (sometimes referred to as Arrestin 3).

The term " β arrestin" (or " β arrestin") as used herein is intended to encompass all types of β arrestin, including but not limited to β arrestin 1 and β arrestin 2.

The phrases "concurrent administration," "administration in combination, if "simultaneous administration" or "administered simultaneously" as used herein,

interchangeably mean that the compounds are administered at the same point in time or immediately following one another. In the latter case, the two compounds are administered at times sufficiently close that the results observed are indistinguishable from those achieved when the compounds are administered at the same point in time.

5 As used herein, exogenous or heterologous DNA (or RNA) refers to DNA (or RNA) which has been introduced into a cell (or the cell's ancestor) through the efforts of humans. Such heterologous DNA may be a copy of a sequence which is naturally found in the cell being transformed, or a sequence which is not naturally found in the cell being transformed, or fragments thereof.

10 As used herein, the term 'gene' refers to a DNA sequence that incorporates (1) upstream (5') regulatory signals including a promoter, (2) a coding region specifying the product, protein or RNA of the gene, (3) downstream (3') regions including transcription termination and polyadenylation signals and (4) associated sequences required for efficient and specific expression.

15 Use of the phrase "substantial sequence similarity" in the present specification refers to DNA, RNA or amino acid sequences which have slight and non-consequential sequence variations from a sequence of interest, and are considered to be equivalent to the sequence of interest. In this regard, "slight and non-consequential sequence variations" mean that "similar" sequences (i.e., sequences that have substantial sequence similarity) 20 will be functionally equivalent. Functionally equivalent sequences will function in substantially the same manner to produce substantially the same compositions.

As used herein, a "native DNA sequence" or "natural DNA sequence" means a DNA sequence which can be isolated from non-transgenic cells or tissue. Native DNA sequences are those which have not been artificially altered, such as by site-directed 25 mutagenesis. Once native DNA sequences are identified, DNA molecules having native DNA sequences may be chemically synthesized or produced using recombinant DNA procedures as are known in the art.

As used herein, "a regulatory element" from a gene is the DNA sequence which is necessary for the expression of the gene, such as a promoter. In this invention, the term "operatively linked" means that following such a link a regulatory element can direct the expression of a linked DNA sequence.

5 The term 'promoter' refers to a region of a DNA sequence that incorporates the necessary signals for the efficient expression of a coding sequence. This may include sequences to which an RNA polymerase binds but is not limited to such sequences and may include regions to which other regulatory proteins bind together with regions involved in the control of protein translation and may include coding sequences. Suitable
10 promoters will be apparent to those skilled in the art, and will vary depending upon the cell in which the DNA is to be expressed. A suitable promoter for use in DNA constructs encoding a β arrestin/detectable molecule construct may be a promoter naturally found in the cell in which expression is desired; optionally, the promoter of the β arrestin within the construct may be utilized. Both inducible and constitutive promoters are contemplated
15 for use in the present invention.

DNA Constructs

DNA constructs, or "expression cassettes," of the present invention include, 5' to 3' in the direction of transcription, a promoter, a DNA sequence operatively associated with the promoter, and, optionally, a termination sequence including stop signal for RNA
20 polymerase and a polyadenylation signal for polyadenylase. All of these regulatory regions should be capable of operating in the cell to be transformed. Suitable termination signals for a given DNA construct will be apparent to those of skill in the art.

The term "operatively associated," as used herein, refers to DNA sequences on a single DNA molecule which are associated so that the function of one is affected by the
25 other. Thus, a promoter is operatively associated with a DNA when it is capable of affecting the transcription of that DNA (i.e., the DNA is under the transcriptional control of the promoter). The promoter is said to be "upstream" from the DNA, which is in turn said to be "downstream" from the promoter.

The present inventors have determined that β arrestin redistribution from the cytosol to the plasma membrane occurs in response to agonist activation of GPCRs. The present inventors demonstrated a common role for β arrestin in agonist-mediated signal transduction termination following agonist activation of receptors. The present inventors have devised convenient methods of assaying agonist stimulation of GPCRS in vivo and in vitro in real time. Although the pharmacology of members of the GPCR superfamily differs, the methods of the present invention utilize β arrestin translocation to provide a single-step, real-time assessment of GPCR function for multiple, distinct members of the GPCR superfamily. The present methods may additionally be utilized in studying and understanding the mechanisms of actions of various therapeutic agents. The present inventors have determined that a protein conjugate or chimera comprising an arrestin molecule and a detectable molecule (such as Green Fluorescent Protein) is useful in such methods of assaying in vivo GPCR activity.

Due to the therapeutic importance of GPCRs, methods for the rapid screening of compounds for GPCR ligand activity are desirable. Additionally, methods of screening orphan GPCRs for interactions with known and putative GPCR ligands assist in characterizing such receptors. Optical methods are available for studying labelled protein dynamics in intact cells, including video microscopy, fluorescence recovery after photobleaching, and resonance energy transfer. However, such methods are of limited usefulness in labeling GPCRs for study, due to the relatively low level of GPCR expression and the alterations in receptor function that can occur after tagging or labeling of the receptor protein. Radiolabeling or fluorescent labeling of test ligands has also been utilized in screening for GPCR ligands. See, e.g. Atlas et al., *Proc. Natl. Acad. Sci. USA* 74:5490 (1977); U.S. Pat. No. 5,576,436 to McCabe et al. (all patents cited herein are incorporated herein in their entirety). The introduction of foreign epitopes into receptor cDNA to produce hybrid GPCRs is now a standard technique, and enhances detection of GPCRs by monoclonal antibody technology. However, such techniques are limited in their applicability to living cells. U.S. Pat. No. 5,284,746 to

Sledziewski describes yeast-mammalian hybrid GPCRs and methods of screening for GPCR ligands using such hybrid receptors. U.S. Pat. No. 5,482,835 to King et al. describes methods of testing in yeast cells for ligands of mammalian GPCRs. However, application of these techniques to the study or identification of orphan GPCRs requires prior knowledge of ligands or signal transduction events and are therefor not generally applicable or universal.

Phosphorylation of GPCRs is a mechanism leading to desensitization of the receptors; receptors that have been continuously or repeatedly stimulated lose responsiveness, whereas the responses of other receptors remain intact. See Harden, *Pharmacol. Rev.* 35:5 (1983); Benovic et al., *Annu. Rev. Cell. Biol.* 4:405(1988). In a variety of cells, specific kinases have evolved for specific GPCRs. Desensitization occurs via the following pathway: agonist occupancy of the receptor transforms the receptor into an appropriate substrate for an associated kinase; β arrestin binds to the kinase phosphorylated receptor and prevents subsequent interaction with the appropriate G-protein, as well as initiating both internalization and resensitization processes. Ferguson et al, *Science*, 271:363 (1996); Lohse et al., *Science* 248:1547 (1990). β arrestin dependent desensitization is induced only when the GPCR is activated by ligand binding, and is an example of homologous desensitization (i.e., the ligand desensitizes only its target receptors). Lohse et al. (1990) and Attramadal et al., *J. Biol. Chem.* 267:17882 (1992) provide cDNA and amino acid sequences of β arrestin. Various isoforms of β arrestin are known; as used herein, β arrestin refers to all such isoforms of β arrestin, proteins having substantial sequence similarity thereto which are functional β arrestins, and functional fragments thereof. Functional fragments of β arrestin, its isoforms and analogs, may be determined using techniques as known in the art.

Molecules detectable by spectroscopic, photochemical, biochemical, immunochemical, electrical and optical means are known. Optically detectable molecules include fluorescent labels, such as commercially available fluorescein and Texas Red. Detectable molecules useful in the present invention include any biologically compatible

molecule which may be conjugated to a β arrestin protein without compromising the ability of β arrestin to interact with the GPCR system, and without compromising the ability of the detectable molecule to be detected. Conjugated molecules (or conjugates) of β arrestin and detectable molecules (which also may be termed 'detectably

5 labelled β arrestins') are thus useful in the present invention. Preferred are detectable molecules capable of being synthesized in the cell to be studied (e.g., where the cell can be transformed with heterologous DNA so that the β arrestin-detectable molecule chimera is produced within the cell). Particularly preferred are those detectable molecules which are inherently fluorescent in vivo. Suitable detectable molecules must be able to be
10 detected with sufficient resolution within a cell that translocation of β arrestin from the cytosol to the cell membrane in response to agonist binding to GPCR can be qualitatively or quantitatively assessed. Molecules detectable by optical means are presently preferred.

Fusion proteins with coding sequences for β -galactosidase, firefly luciferase, and bacterial luciferase have been used in methods of detecting gene expression and protein
15 interactions in cells. However, these methods require exogenously-added substrates or cofactors. In the methods of the present invention, an inherently fluorescent marker molecule is preferred, such as GFP, since detection of such a marker intracellularly requires only the radiation by the appropriate wavelength of light and is not substrate limited.

20 Green Fluorescent Protein (GFP) was first isolated from the jelly fish *Aequorea victoria*, and has an inherent green bioluminescence that can be excited optically by blue light or nonradiative energy transfer. Sequences of GFP-encoding cDNA and GFP proteins are known; see, e.g., Prasher et al., *Gene*, 111:229 (1992). The crystalline structure of GFP is described in Ormo et al., *Science* 273:1392 (1996). Purified native
25 GFP absorbs blue light (maximally at 395 nm with a minor peak at 470 m) and emits green light (peak emission at 509 nm) (Morise et al, *Biochemistry*, 13:2656 (1974); Ward et al., *Photochem. Photobiol.*, 31:611 (1980)). It has been shown that GFP expressed in prokaryotic and eukaryotic cells produces a strong green fluorescence when excited by

near UV or blue light (see U.S. Pat. No. 5,491,084 to Chalfie and Prasher); as this fluorescence requires no additional gene products from *A. victoria*, chromophore formation is not species specific and occurs either through the uses of ubiquitous cellular components or by autocatalysis. Expression of GFP in *Escherichia coli* results in an easily detected green fluorescence that is not seen in control bacteria. See Chalfie et al., *Science* 263:802 (1994); U.S. Pat. No. 5,491,084. Cells expressing the green-fluorescent proteins may be conveniently separated from those which do not express the protein by a fluorescence-activated cell sorter.

As used herein, Green Fluorescent Protein refers to the various naturally occurring forms of GFP which can be isolated from natural sources, as well as artificially modified GFPs which retain the fluorescent abilities of native GFP. As discussed in Ormo et al., *Science* 273:1392 (1996), various mutants of GFP have been created with altered excitation and emission maxima. Two characteristics of wild-type GFP which affect its usefulness in mammalian cell lines are the need to excite it at UV wavelengths to obtain a maximal fluorescent signal, and decreased fluorescence at temperatures over 23.degree. C. However, the S65T/GFP mutant overcomes these limitations. Heim et al., *Proc. Natl. Acad. Sci. USA* 91:12501 (1994). Additional alterations in the GFP protein sequence which provide inherently fluorescent, biologically compatible molecules will be apparent to those in the art; sequence alterations may be made to alter the solubility characteristics of the protein, its excitation wavelength, or other characteristics, while retaining useful fluorescent properties. See, e.g. U.S. Pat. No. 5,625,048 to Tsien and Heim; WO 9711091 (Bjorn, Poulsen, Thastrup and Tullin); WO 9627675 (Haseloff, Hodge, Prasher and Siemering); WO 9627027 (Ward); WO 9623898 (Bjorn et al.); WO 9623810 (Heim and Tsien); WO 9521191 (Chalfie and Ward).

Cells useful in the methods of the present invention include eukaryotic and prokaryotic cells, including but not limited to bacterial cells, yeast cells, fungal cells, insect cells, nematode cells, plant or animal cells. Suitable animal cells include, but are not limited to HEK cells, HeLa cells, COS cells, and various primary mammalian cells.

Cells contained in intact animals, including but not limited to nematodes, zebrafish (and other transparent or semi-transparent animals) and fruitflies, may also be used in the methods of the present invention. An animal model expressing a β arrestin-detectable molecule fusion protein throughout its tissues, or within a particular organ or tissue type, will be useful in studying cellular targets of known or unknown GPCR ligands.

Cells useful in the present methods include those which express a known GPCR or a variety of known GPCRs, or which express an unknown GPCR or a variety of unknown GPCRs. As used herein, a cell which expresses a GPCR is one which contains that GPCR as a functional receptor in its membrane; the cells may naturally express the GPCR(s) of interest, or may be genetically engineered to express the GPCR(s) of interest. As used herein, an 'unknown' or 'orphan' receptor is one whose function is unknown, and/or whose ligands are unknown.

The present experiments

Green fluorescent protein (GFP) has been used to study protein-protein interactions in living cells. See Kaether & Gerdes, *FEBS Lett.* 369:267 (1995); Olson et al., *J. Cell. Biol.* 130:639 (1995). Green fluorescent protein (GFP) is useful as a reporter molecule for fusion proteins due to its inherent fluorescence and its folding, which apparently isolates it from its conjugated partner. Prasher et al., *Gene* 111:229 (1992); Ormo et al., *Science* 273:1392 (1996). For example, a seven transmembrane protein as complex as the β 2AR, which is three times larger than GFP, exhibits normal biochemistry after GFP conjugation to its C-terminus. Barak et al., *Mol. Pharmacol.* 51:177 (1997).

The present inventors established that a fusion protein consisting of a β arrestin molecule (β arrestin2) conjugated to a GFP at its C-terminus (β arr2-GFP, FIG. 1) is expressed in cells and is biologically active. The β arr2-GFP fusion protein is approximately 50% larger than β arrestin2, and this size increase is reflected by its slower migration on SDS-Page (FIG. 2A). The left lane of FIG. 2A, exposed to an antibody against β arrestin, shows that β arr2-GFP runs more slowly than endogenous β arrestin2

(highlighted middle band). The right lane of FIG. 2A, treated with a monoclonal anti-GFP antibody, demonstrates that the slower band does indeed contain GFP. β 2AR normally sequesters poorly in COS cells, and this has been correlated to the relatively poor expression of endogenous β arrestins in COS cells. Menard et al., *Mol. Pharmacol.* 51:800 (1997); Zhang et al., *J. Biol. Chem.* 271:18302 (1996). Overexpression of exogenous β arrestin enhances β 2AR sequestration in these cells; similarly, as shown herein, β arr2-GFP overexpression in COS cells augmented β 2AR internalization (FIG. 2B), demonstrating that β arr2-GFP is biologically active and equivalent to native β arrestin.

Biochemical evidence indicates that β arrestins are predominantly cytosolic proteins. Ferguson et al., *Can. J. Physiol. Pharmacol.* 74:1095 (1996). The present inventors, using confocal microscopy of β arr2-GFP in HEK-293 cells (FIG. 3A, left panel), confirmed that β arr2-GFP is distributed throughout the cytosol and excluded from the nucleus. The present data also establish for the first time that β arrestin is not predominantly compartmentalized at the plasma membrane in the absence of agonist but that, upon addition of saturating concentrations of an agonist to the cell medium, β arrestin is translocated from cell cytosol to cell membrane. Where β arrestin is conjugated to an optically detectable molecule such as GFP, as shown herein, a rapid and readily observable optical enhancement of the membrane and a concomitant loss of cytosolic optical signals occurs (see FIGS. 3A and 3B, where membrane fluorescence is enhanced and cytosol fluorescence is decreased due to translocation of the β arrestin-GFP chimera).

To investigate whether the intracellular translocation of β arrestin targeted binding sites in the plasma membrane other than the β 2AR, the present inventors first crosslinked the receptors using monoclonal antibodies. As reported herein and shown in FIG. 4, the geometry of the agonist-induced time dependent translocation of β arrestin to the plasma membrane mimicked the distribution of pre-aggregated β 2ARs, indicating that the targeted site of β arrestin is indeed β 2AR or an associated component.

It has been postulated that phosphorylation of GPCRs by GRKs facilitates desensitization by increasing their affinity for β arrestins. Gurevich et al, *J. Biol. Chem.* 268:16879 (1993); Gurevich et al., *J. Biol. Chem.* 268:11628 (1993). When expressed in HEK-293 cells and exposed to agonist, mutant Y326A- β 2ARs are not significantly phosphorylated by endogenous GRKs (Ferguson et al., *J. Biol. Chem.*, 270:24782 (1995). Therefore, the present inventors utilized this mutant receptor to investigate the above question of β arrestin affinity in vivo. Y326A- β 2AR was cotransfected with β arr2-GFP into HEK cells in the absence and presence of co-transfected GRK. If the above hypothesis were true, reversal of phosphorylation impairment by overexpressed GRKs would result in a noticeable difference in β arr2-GFP translocation. As reported herein, without added GRK, β arr2-GFP translocation in response to agonist proceeded poorly; with the addition of GRK, β arr2-GFP translocation to the plasma membrane was much more robust (FIG. 5), indicating the importance of phosphorylation to β arrestin activity.

The present inventors determined that translocation of β arrestin from the cell cytosol to the cell membrane is an indicator of agonist stimulation of GPCR activity, and that a chimeric protein comprising β arrestin and the detectable molecule GFP was capable of detectably displaying the real-time translocation of β arrestin in response to agonist activation of GPCRs.

The results presented herein establish that β arrestin targets GPCRs or an associated molecule following agonist binding and receptor phosphorylation. These data demonstrate a biological behavior for β arrestin that has only been postulated from biochemical studies, and characterize for the first time how β arrestin compartmentalization changes after initiation of receptor signal transduction. Agonist activation of a GPCR ultimately culminates in the association of β arrestins with GPCRs, thus the visualization of the agonist mediated β arrestin translocation process provides a universal indicator of GPCR activation.

The present inventors have demonstrated that GPCR signal transduction induces a rapid, substantial increase in the relative and absolute amount of plasma membrane bound β arrestin. The agonist-mediated redistribution of β arrestin coupled to a detectable molecule provides an optical amplification of the extracellular signals transduced by GPCRs, and this occurs simultaneous with, or within the same time frame as, the chemical amplification normally provided by second messenger cascades. Chimeras of β arrestin and a detectable molecule are useful for the study of β arrestin kinetics and GPCR related behavior such as endocytosis. Additionally, such chimeras are useful as biosensors for signaling when GPCRs become activated, and provide methods of screening compounds for GPCR activity, and screening orphan GPCRs for ligand responsiveness. In addition, the ability of co-transfected GRKs to enhance both the rate and extent of β arrestin translocation indicate that the present methods and constructs can also be used to monitor GRK activity, as well as monitor drugs, proteins and compounds for activation or inhibition of the GRK/ β arrestin process.

The present invention provides a method for screening compounds for GPCR agonist activity, comprising: a) providing a cell expressing a known or unknown GPCR and containing a chimeric protein comprising a β arrestin protein and a visually detectable protein; b) exposing the cell to a test compound; and c) detecting translocation of the detectable molecule from the cytosol of the cell to the membrane edge of the cell; where translocation of the detectable molecule from the cytosol to the membrane edge of the cell indicates activation of the GPCR and, accordingly, the GPCR activating effect of the test compound. Translocation of the chimeric protein is evidenced by an increase in the intensity of detectable signal located at the membrane edge (and/or a decrease in the cytosol), where the change occurs after exposure to the test compound. Translocation may thus be detected by comparing changes in the detectable signal in the same cell over time (i.e., pre and post test compound exposure). Alternatively, a test cell may be compared to a control cell (no exposure to test compound), or a test cell may be compared to a pre-established standard. If a known agonist is available the present methods can be

used to screen for and study GPCR antagonists. Additionally, the membrane association of β arrestin should be increased by expression of an excess of receptor or by a constitutively active GPCR that undergoes phosphorylation by GRKs even in the absence of agonist. Therefore, the present methods can be used to monitor for inverse agonists of GPCRs.

Methods of detecting the intracellular translocation of the chimeric protein will depend on the particular detectable protein utilized; one skilled in the art will be able to readily devise detection methods suitable for particular detectable molecules, given the teachings of the present specification and knowledge in the art. In a preferred embodiment, the visually detectable protein is a green-fluorescent protein (GFP) as discussed below.

The methods of the present invention provide easily detectable results. The translocation of β arrestin coupled to a detectable molecule such as GFP, in response to GPCR activation, results in a relative enhancement of the detectable signal at the cell edge (i.e., at the cell membrane). In addition, the concomitant decrease in detectable signal from the cell cytosol means that 'background noise' (detectable signals which do not change in response to GPCR activation) is minimized. In certain cells, activation of GPCRs will result in essential clearing of detectable signal from the cytosol, and a 100-fold increase (or more) in the detectable signal at the cell membrane. In the present methods, it is preferred that the detectable signal at the membrane edge increase, after GPCR activation, at least two-fold, more preferably at least 3-fold, and more preferably at least 5-fold or at least ten-fold.

As used herein, the introduction of a chimeric protein into a cell may be accomplished by introducing into the cell (or the cell's ancestor) a nucleic acid (e.g., DNA or RNA) sequence or construct encoding the chimeric protein, and culturing the cell in an environment which allows expression of the chimeric protein. Introduction of nucleic acids encoding the chimeric protein, or introduction of the protein itself, into a cell may

be carried out by any of the many suitable methods which are known in the art, including transfection, electroporation, microinjection, and liposome delivery.

The present invention provides a DNA construct comprising a promoter, DNA encoding a β arrestin protein operatively associated therewith, and DNA encoding a visually detectable marker protein operatively associated therewith. The promoter is operatively associated with the encoding DNA; DNA encoding β arrestin may be 5' from DNA encoding the visually detectable marker, or vice versa. In a preferred embodiment, the DNA encoding a visually detectable marker encodes a green-fluorescent protein (GFP). Vectors comprising such DNA constructs are a further aspect of the present invention.

The present invention further provides conjugates (such as chimeric proteins or fusion proteins) which comprise a β arrestin protein and a visually detectable protein. In a preferred embodiment, the visually detectable protein is a green-fluorescent protein (GFP).

The present invention further provides a cell comprising a DNA molecule, which DNA molecule comprises, in the 5' to 3' direction, a promoter, DNA encoding a β arrestin protein operatively associated therewith, and DNA encoding a visually detectable marker protein operatively associated therewith. In a preferred embodiment, the DNA encoding a visually detectable marker encodes a green-fluorescent protein (GFP).

The cells of the present invention may be used to detect the presence of specific molecules in various kinds of samples such as, e.g., aqueous samples, biological samples (for example blood, urine or saliva), environmental samples, or industrial samples. In such uses, the cells contain a GPCR whose agonists are known. Activation of the GPCR and the concomitant translocation of the detectable signal from the cytosol to the membrane edge indicates the presence of the agonist for the GPCR. A cell used in such a method may contain only a single type of known GPCR, or a variety of known GPCRs. Such detection will be useful for medical and veterinary diagnostic purposes; industrial

purposes; and screening for drugs or chemicals of abuse or biological toxins that affect GPCR-mediated signal transduction.

The cells of the present invention may be deposited on, affixed to, supported by, or immobilized on a substrate. The substrate may be of any suitable material which is not harmful or detrimental to the living cells deposited thereon, i.e., which is bio-compatible with the living material deposited thereon. The substrate may be rigid, semi-rigid or flexible; and may be opaque, transparent, or semi-transparent. The size, geometry and other physical characteristics of the substrate will be dictated by the intended use, as will be apparent to one skilled in the art. Suitable substrates include, but are not limited to, plastics, glass, ceramics, silica, biocompatible monomer and polymer compositions, semiconductor materials, fiber optic materials, polystyrene, membranes, sephadex, and bio-organic materials. Examples of biocompatible materials are provided in U.S. Pat. Nos. 5,578,079; 5,575,997 and 5,582,834 to Leung and Clark; and U.S. Pat. No. 5,522,896 to Prescott.

The present invention further provides methods for screening for the presence of a GPCR agonist in a solution which comprises: a) providing a cell expressing a known or unknown GPCR and containing a chimeric protein comprising a β arrestin protein and a visually detectable protein; b) exposing the cell to a test solution; and c) detecting translocation of the detectable molecule from the cytosol of the cell to the membrane edge of the cell; where translocation of the detectable molecule from the cytosol to the membrane edge of the cell indicates activation of the GPCR and, accordingly, the GPCR agonist effect of the test solution. Translocation of the chimeric protein is evidenced as discussed above.

The present invention further provides methods for screening for the presence of a GPCR antagonist in a solution which comprises: a) providing a cell expressing a GPCR and containing a chimeric protein comprising a β arrestin protein and a visually detectable protein; b) exposing the cell to a test compound; then c) exposing the cell to a known agonist to the GPCR expressed in the cell; and d) detecting translocation of the detectable

molecule from the cytosol of the cell to the membrane edge of the cell. If the test compound contains an antagonist, translocation of the detectable molecule will be delayed for a period of time corresponding to duration of antagonist action on the receptor (which time period will vary depending on the antagonist and/or the receptor).

- 5 Translocation of the detectable molecule from the cytosol to the membrane edge of the cell indicates activation of the GPCR by the agonist. Accordingly, when translocation does not occur or is delayed (compared to that which would occur in the absence of test compound), the test compound contains an antagonist to the GPCR. Absence or delay of translocation may be assessed by comparison to a control cell (not exposed to test
10 compound) or to a predetermined standard. Translocation of the chimeric protein is evidenced as discussed above. Exposure to the test compound and the known agonist may occur at essentially the same time, or exposure to the agonist may occur subsequent to exposure to the test compound. As used herein, subsequent exposure refers to exposure within the time period during which a potential antagonist would be expected to
15 be interacting with the GPCR (i.e., binding to or bound to the GPCR).

The present invention further provides methods for screening a cell for the presence of a GPCR, comprising: a) providing a test cell; b) introducing into the test cell a chimeric protein comprising a β arrestin protein and a visually detectable protein; and then c) exposing the cell to a test solution containing a known agonist to a GPCR; and
20 d) detecting translocation of the detectable molecule from the cytosol of the cell to the membrane edge of the cell; where translocation of the detectable molecule from the cytosol to the membrane edge of the cell indicates activation of a GPCR and, accordingly, that the test cell contains such a GPCR. Translocation of the chimeric protein is evidenced as discussed above.

25 The present invention further provides methods for screening a cell population for the presence of cells containing GPCRs, comprising: a) providing a population of test cells, said test cells containing chimeric proteins comprising a β arrestin protein and a visually detectable protein; and then b) exposing the cell population to a test solution

containing an agonist to a GPCR; and d) detecting those cells in which translocation of the detectable molecule from the cytosol of the cell to the membrane edge of the cell occurs; where translocation of the detectable molecule from the cytosol to the membrane edge of a cell indicates activation of a GPCR and, accordingly, that the cell in question contains a GPCR. Translocation of the chimeric protein is evidenced as discussed above. Populations of cells to be screened include a collection of individual cells, a tissue comprising a plurality of similar cells, an organ comprising a plurality of related cells, or an organism comprising a plurality of tissues and organs.

As used herein, 'exposing' a cell to a test compound or solution means bringing the cell exterior in contact with the test compound or solution. Where the test compound or solution is being screened for GPCR ligand activity, exposure is carried out under conditions that would permit binding of a GPCR ligand to a receptor expressed in that cell. As used herein, 'translocation' of β arrestin refers to movement of the β arrestin molecule from one area of the cell to another.

The present methods may further be used to assess or study the effects of any molecule in the GPCR pathway which exerts its effect upstream of β arrestin binding (i.e., prior to β arrestin binding to the phosphorylated GPCR). Thus the present invention provides methods for assessing GPCR pathway functions in general. As used herein, the GPCR pathway refers to the series of events which starts with agonist activation of a GPCR followed by desensitization of the receptor via G protein-coupled receptor kinase (GRK) phosphorylation and β arrestin binding.

In a broad sense the present invention thus provides a method of screening test compounds and test conditions for the ability to affect (activate or inhibit, enhance or depress) a GPCR pathway, and provides methods of assessing GPCR pathway function in a cell in general. In the present methods, the extent of translocation of β arrestin is indicated by the degree of detectable changes in the cell; the extent of β arrestin translocation is an indicator of the extent of GPCR pathway completion. The relative

extent of translocation under varied test conditions may be compared, or a test condition may be compared to a control condition or to a predetermined standard.

For example, the specificity and effects of various kinases (including those known to interact with GPCR pathways and those not previously known to interact with GPCRs) for a specific GPCR or a group of GPCRs may be assessed by providing a test kinase to a test cell expressing a GPCR and containing a detectable β arrestin molecule, exposing the cell to a GPCR agonist, and assessing the translocation of detectable β arrestin from the cell cytosol to the cell membrane (see Example 7 herein). Translocation of the β arrestin to the cell membrane indicates that the test kinase, in response to agonist occupancy of the receptor, is able to bind to and phosphorylate the receptor, so that β arrestin will then bind to the kinase phosphorylated receptor and prevent subsequent interaction with the appropriate G-protein. In similar ways, the function of altered, recombinant or mutant kinases may be assessed; compounds may be screened for the ability to activate or inhibit the GPCR pathway, G protein-coupled receptor kinases, or β arrestin binding; and the function of G-proteins may be assessed. For example, the following test conditions may be assessed using methods as described herein: the effects of G-proteins (including natural, heterologous, or artificially altered G-proteins) within the test cell; exposure of the test cell to known or putative GPCR ligands; and co-expression of a second receptor in the test cell expressing a GPCR.

Still further, the present methods allow the screening of β arrestins (naturally occurring, artificially introduced, or altered, mutant or recombinant) for the ability to bind to a phosphorylated GPCR. In such methods, the test β arrestin is conjugated to a detectable molecule such as GFP, and is placed within a cell containing a GPCR. The cell is exposed to a known agonist of the GPCR, and translocation of the detectable molecule from the cytosol of the cell to the membrane edge of the cell is detected. The translocation of the detectable molecule indicates that the test β arrestin protein is able to bind to the phosphorylated GPCR. As in other methods of the present invention, the

translocation may be compared to a control cell containing a known β arrestin, or to a predetermined standard.

G Protein Coupled Receptors

GPCRs suitable for use in the present methods are those in which agonist binding induces G protein-coupled receptor kinase (GRK) phosphorylation; translocation of arrestin from the cytosol of the cell to the cell membrane subsequently occurs. As it is believed that virtually all members of the GPCR superfamily desensitize via this common mechanism, examples of suitable types of GPCRs include but are not limited to beta and alpha adrenergic receptors; GPCRs binding neurotransmitters (such as dopamine); GPCRs binding hormones; the class of odorant receptors (taste, smell and chemotactic receptors as found in nasal mucosa and the tongue, and on sperm, egg, immune system cells and blood cells); the class of type II GPCRs including secretin, glucagon, and other digestive tract receptors; light-activated GPCRs (such as rhodopsin); and members of the type III family of GPCRs which include but are not limited to metabotropic glutamate receptors and GABA.sub.B receptors. In addition to naturally occurring GPCRs, GPCRs may be specifically engineered or created by random mutagenesis. Such non-naturally occurring GPCRs may also be utilized in and screened by the present methods. The present methods may be utilized with any membrane receptor protein in which agonist binding results in the translocation of β arrestin. Such receptors include growth factors that signal through G proteins.

Automated Screening Methods

The methods of the present invention may be automated to provide convenient, real time, high volume methods of screening compounds for GPCR ligand activity, or screening for the presence of a GPCR ligand in a test sample. Automated methods are designed to detect the change in concentration of labelled β arrestin at the cell membrane and/or in the cytosol after exposure to GPCR agonist. The alteration of β arrestin distribution can be detected over time (i.e., comparing the same cell before and after exposure to a test sample), or by comparison to a control cell which is not exposed to the

test sample, or by comparison to pre-established indicia. Both qualitative assessments (positive/negative) and quantitative assessments (comparative degree of translocation) may be provided by the present automated methods, as will be apparent to those skilled in the art.

5 It is thus a further object of the present invention to provide methods and apparatus for automated screening of GPCR activity, by detecting the translocation of detectably labeled β arrestin from cell cytosol to cell membrane in response to agonist activation of GPCRs. The translocation may be indicated by an alteration in the distribution of a detectable signal within a cell over time, between a test cell and a control
10 cell, or by comparison to previously established parameters. In particular, according to one embodiment of the present invention, a plurality of cells expressing GPCRs and containing chimeric proteins comprising a detectable molecule and a β arrestin molecule are provided. Indicia of the distribution of the detectable molecules are then measured using conventional techniques. In various embodiments, (a) measurement of optical
15 indicia occurs before and after the addition of a test sample to a cell, and the time point measurements are compared; (b) optical indicia are measured in a test cell exposed to a test sample and in a non-exposed control cell, and these measurements are compared; and (c) measurement of a test cell after addition of a test sample is compared to preestablished parameters. The optical indicia being measured may be fluorescence signals (e.g.,
20 fluorescence intensities) if the detectable molecule of the chimeric β arrestin protein is a fluorescent indicator such as GFP. Other optical indicia that are suitable for real-time measurement may also be used, as will be apparent to those skilled in the art.

An embodiment of the present invention includes an apparatus for determining GPCR response to a test sample. This apparatus comprises means, such as a fluorescence
25 measurement tool, for measuring indicia of the intracellular distribution of detectable β arrestin proteins in at least one test cell, and optionally also in a control or calibration cell. Measurement points may be over time, or among test and control cells. A computer program product controls operation of the measuring means and performs numerical

operations relating to the above-described steps. The preferred computer program product comprises a computer readable storage medium having computer-readable program code means embodied in the medium. Hardware suitable for use in such automated apparatus will be apparent to those of skill in the art, and may include computer controllers,

5 automated sample handlers, fluorescence measurement tools, printers and optical displays. The measurement tool may contain one or more photodetectors for measuring the fluorescence signals from samples where fluorescently detectable molecules are utilized in the detectable β arrestin construct. The measurement tool may also contain a computer-controlled stepper motor so that each control and/or test sample can be

10 arranged as an array of samples and automatically and repeatedly positioned opposite a photodetector during the step of measuring fluorescence intensity.

The measurement tool is preferably operatively coupled to a general purpose or application specific computer controller. The controller preferably comprises a computer program product for controlling operation of the measurement tool and performing

15 numerical operations relating to the above-described steps. The controller may accept set-up and other related data via a file, disk input or data bus. A display and printer may also be provided to visually display the operations performed by the controller. It will be understood by those having skill in the art that the functions performed by the controller may be realized in whole or in part as software modules running on a general purpose

20 computer system. Alternatively, a dedicated stand-alone system with application specific integrated circuits for performing the above described functions and operations may be provided.

As provided above, the indicia of β arrestin distribution may take the form of fluorescent signals, although those skilled in the art will appreciate that other indicia are

25 known and may be used in the practice of the present invention, such as may be provided by labels that produce signals detectable by fluorescence, radioactivity, colorimetry, X-ray diffraction or absorption or magnetism. Such labels include, for example, fluorophores,

chromophores, radioactive isotopes (e.g., ³²P or ¹²⁵I) and electron-dense reagents.

The expression or transcription cassette may be provided in a DNA construct which also has at least one replication system. For convenience, it is common to have a replication system functional in *Escherichia coli*, such as Co1E1, pSC101, pACYC184, or the like. In this manner, at each stage after each manipulation, the resulting construct may be cloned, sequenced, and the correctness of the manipulation determined. In addition, or in place of the *E. coli* replication system, a broad host range replication system may be employed, such as the replication systems of the P-1 incompatibility plasmids, e.g., pRK290. In addition to the replication system, there will frequently be at least one marker present, which may be useful in one or more hosts, or different markers for individual hosts. That is, one marker may be employed for selection in a prokaryotic host, while another marker may be employed for selection in a eukaryotic host. The markers may be protection against a biocide, such as antibiotics, toxins, heavy metals, or the like; may provide complementation, by imparting prototrophy to an auxotrophic host; or may provide a visible phenotype through the production of a novel compound in the plant.

The various fragments comprising the various constructs, expression cassettes, markers, and the like may be introduced consecutively by restriction enzyme cleavage of an appropriate replication system, and insertion of the particular construct or fragment into the available site. After ligation and cloning the DNA construct may be isolated for further manipulation. All of these techniques are amply exemplified in the literature as exemplified by J. Sambrook et al., Molecular Cloning, A Laboratory Manual (2d Ed. 1989)(Cold Spring Harbor Laboratory).

Arrestin Knockout Mice

The production of β arrestin knockout mice can be carried out in view of the disclosure provided herein and in light of techniques known to those skilled in the art, such as described in U.S. Patents Nos. 5,767,337 to Roses et al.; 5,569,827 to Kessous-

Elbaz et al.; and 5,569,824 to Donehower et al. (the disclosures of which applicants specifically intend to be incorporated by reference herein in their entirety); and A. Harada et al., *Nature* **369**, 488 (1994). Particularly preferred mice for carrying out the present invention are also disclosed below.

5 **1. Assay techniques.** The step of determining whether or not β arrestin binding to the phosphorylated μ opioid receptor is inhibited by the test compound may be carried out by any suitable technique, including *in vitro* assay and *in vivo* assay (e.g., in a cell that contains the β arrestin and the phosphorylated μ opioid receptor). A particularly suitable technique for *in vivo* assay is as described previously. In general, this technique involves
10 providing a cell that expresses μ opioid receptor as a G-protein coupled receptor, and contains the β arrestin protein conjugated to an optically detectable molecule (e.g., green fluorescent protein). The test compound is then introduced into the cell (e.g., by microinjection, by electroporation, by suspending the cell in an aqueous solution that contains the test compound, by contacting the cell to liposomes that contain the test
15 compound, by insertion of a heterologous nucleic acid into the cell that encodes and expresses the test compound, etc.). Translocation of the molecule from the cytosol of the cell to the membrane edge of the cell is then monitored or examined, with the inhibition of such translocation indicating that the test compound inhibits the binding of β arrestin to the phosphorylated μ opioid receptor. If desired, phosphorylation of the μ opioid receptor
20 can be induced or enhanced by any suitable means, such as contacting a μ opioid receptor agonist such as morphine to the cell in an amount effective to induce phosphorylation (e.g., by adding the agonist to the culture medium or liquid medium in which the cell is contained). The cell is preferably a mammalian cell, but any suitable cell can be employed, including bacterial cells, yeast cells, fungal cells, plant cells, and other animal
25 cells, so long as they express μ opioid receptor and phosphorylate, or can be induced to phosphorylate, the same, and contain the desired β arrestin protein coupled to an optically detectable molecule (e.g., either by exogenous introduction or expression of the β arrestin

conjugate therein). Any suitable β arrestin may be employed as described above, with β arrestin-2 being preferred.

2. Test compounds. The present invention can be used with test compounds (or "probe molecules"), or libraries (where groups of different probe molecules are employed), of any type. In general, such probe molecules are organic compounds, including but not limited to oligomers, non-oligomers, or combinations thereof. Nonoligomers include a wide variety of organic molecules, such as heterocyclics, aromatics, alicyclics, aliphatics and combinations thereof, comprising steroids, antibiotics, enzyme inhibitors, ligands, hormones, drugs, alkaloids, opioids, benzodiazepenes, terpenes, porphyrins, toxins, catalysts, as well as combinations thereof. Oligomers include peptides (that is, oligopeptides) and proteins, oligonucleotides (the term oligonucleotide also referred to simply as "nucleotide", herein) such as DNA and RNA, oligosaccharides, polylipids, polyesters, polyamides, polyurethanes, polyureas, polyethers, poly (phosphorus derivatives) such as phosphates, phosphonates, phosphoramides, phosphonamides, phosphites, phosphinamides, etc., poly (sulfur derivatives) such as sulfones, sulfonates, suffites, sulfonamides, sulfenamides, etc., where for the phosphorous and sulfur derivatives the indicated heteroatom for the most part will be bonded to C, H, N, O or S, and combinations thereof. Numerous methods of synthesizing or applying such probe molecules on solid supports (where the probe molecule may be either covalently or non-covalently bound to the solid support) are known, and such probe molecules can be made in accordance with procedures known to those skilled in the art. See, e.g., U.S. Patent No. 5,565,324 to Still et al., U.S. Patent No. 5,284,514 to Ellman et al., U.S. Patent No. 5,445,934 to Fodor et al. (the disclosures of all United States patents cited herein are to be incorporated herein by reference in their entirety).

3. Pain control and active compounds. As noted above, the present invention provides a method of controlling pain in a subject, comprising inhibiting β arrestin binding to the phosphorylated μ opioid receptor in said subject in an amount effective to

induce or enhance analgesia in the subject. The method may be carried out with or without concurrently administering a μ opioid receptor agonist such as morphine (or other opiate, as described below). When carried out without concurrent administration of μ opioid receptor, the analgesic activity relies upon the activity of endogenous opioid receptor agonists.

The inhibiting of β arrestin binding (preferably β arrestin-2 binding) to phosphorylated μ opioid receptor can be carried out directly or indirectly by any suitable means, including but not limited to knockout of the β arrestin gene as described herein, disabling or downregulating the kinase responsible for phosphorylation of the μ opioid receptor, administration of an antisense oligonucleotide that downregulates expression of the β arrestin, or the administration of an active compound that competitively inhibits binding of the β arrestin to phosphorylated μ opioid receptor (which may be identified by the assay techniques described above). Obviously, functional μ opioid receptor itself must remain in the cells (particularly nerve cells) of the subject so that the primary analgesic activity of the μ opioid receptor agonist can be exerted.

Compounds produced or identified as active compounds by application of the assay procedures described herein to the test compounds or probe molecules described herein are useful *in vitro* and *in vivo* as μ opioid receptor agonists (in that they enhance the activity of opioids, although they do not bind to the same site as an opioid), are useful in enhancing the efficacy, potency, or analgesic activity of μ opioid receptor agonists. Such compounds are also useful *in vivo* in controlling pain in a subject in need thereof. By "controlling pain", "control of pain" and the like herein is meant partially or completely inhibiting a pain response or perception of pain in a subject, and/or partially or fully inducing local or general analgesia in a subject, either alone or in combination with another active agent administered to the subject such as a μ opioid receptor agonist (e.g., morphine). Subjects that may be treated by the compounds identified by the present invention include both human subjects and animal subjects (e.g., dogs, cats, horses, cattle) for veterinary purposes.

Thus, as noted above, further aspects of the present invention include active compounds produced or identified by the methods described hereinabove and pharmaceutical formulations of the same (e.g., said compound in a sterile pyrogen-free saline solution), along with the use of such compounds for the preparation of a medicament for the potentiation of the activity of μ opioid receptor agonists such as morphine, and/or for the control of pain, in a subject in need thereof, either alone or in combination with a μ opioid receptor agonist such as morphine.

In addition to morphine, other μ opioid receptor agonists, typically opiates, that may be used in conjunction with the present invention include, but are not limited to, codeine, oxycodone, hydromorphone, diamorphine, methadone, fentanyl, sufentanil, buprenorphine, meperidine (Demerol®), etc.

The active compounds described above may be combined with a pharmaceutical carrier in accordance with known techniques to provide a pharmaceutical formulation useful carrying out the methods described above. See, e.g., Remington, The Science And Practice of Pharmacy (9th Ed, 1995). In the manufacture of a pharmaceutical formulation according to the invention, the active compound (including the physiologically acceptable salts thereof) is typically admixed with, inter alia, an acceptable carrier. The carrier must, of course, be acceptable in the sense of being compatible with any other ingredients in the formulation and must not be deleterious to the patient. The carrier may be a solid or a liquid, or both, and is preferably formulated with the compound as a unit-dose formulation, for example, a tablet, which may contain from 0.01 or 0.5% to 95% or 99% by weight of the active compound. One or more active compounds may be incorporated in the formulations of the invention, which may be prepared by any of the well known techniques of pharmacy consisting essentially of admixing the components, optionally including one or more accessory ingredients.

The formulations of the invention include those suitable for oral, rectal, topical, buccal (e.g., sub-lingual), parenteral (e.g., subcutaneous, intramuscular, intradermal, or intravenous), topical (i.e., both skin and mucosal surfaces), the most suitable route in any

given case will depend on the nature and severity of the condition being treated and on the nature of the particular active compound which is being used.

Formulations of the present invention suitable for parenteral administration comprise sterile aqueous and non-aqueous injection solutions of the active compound, which preparations are preferably isotonic with the blood of the intended recipient. These preparations may contain anti-oxidants, buffers, bacteriostats and solutes which render the formulation isotonic with the blood of the intended recipient. Aqueous and non-aqueous sterile suspensions may include suspending agents and thickening agents. The formulations may be presented in unit dose or multi-dose containers, for example sealed ampoules and vials, and may be stored in a freeze-dried (lyophilized) condition requiring only the addition of the sterile liquid carrier, for example, saline or water-for-injection immediately prior to use. Extemporaneous injection solutions and suspensions may be prepared from sterile powders, granules and tablets of the kind previously described. For example, in one aspect of the present invention, there is provided an injectable, stable, sterile composition comprising a compound of the present invention+, or a salt thereof, in a unit dosage form in a sealed container. The compound or salt is provided in the form of a lyophilizate which is capable of being reconstituted with a suitable pharmaceutically acceptable carrier to form a liquid composition suitable for injection thereof into a subject. The unit dosage form typically comprises from about 10 mg to about 10 grams of the compound or salt. When the compound or salt is substantially water-insoluble, a sufficient amount of emulsifying agent which is physiologically acceptable may be employed in sufficient quantity to emulsify the compound or salt in an aqueous carrier. One such useful emulsifying agent is phosphatidyl choline.

Formulations suitable for topical application to the skin preferably take the form of an ointment, cream, lotion, paste, gel, spray, aerosol, or oil. Carriers which may be used include petroleum jelly, lanoline, polyethylene glycols, alcohols, transdermal enhancers, and combinations of two or more thereof.

Formulations suitable for transdermal administration may be presented as discrete patches adapted to remain in intimate contact with the epidermis of the recipient for a prolonged period of time. Formulations suitable for transdermal administration may also be delivered by iontophoresis (see, for example, *Pharmaceutical Research* 3(6):318 (1986)) and typically take the form of an optionally buffered aqueous solution of the active compound. Suitable formulations comprise citrate or bis/tris buffer (pH 6) or ethanol/water and contain from 0.1 to 0.2M active ingredient.

Formulations suitable for oral administration may be presented in discrete units, such as capsules, cachets, lozenges, or tablets, each containing a predetermined amount of the active compound; as a powder or granules; as a solution or a suspension in an aqueous or non-aqueous liquid; or as an oil-in-water or water-in-oil emulsion. Such formulations may be prepared by any suitable method of pharmacy which includes the step of bringing into association the active compound and a suitable carrier (which may contain one or more accessory ingredients as noted above). In general, the formulations of the invention are prepared by uniformly and intimately admixing the active compound with a liquid or finely divided solid carrier, or both, and then, if necessary, shaping the resulting mixture. For example, a tablet may be prepared by compressing or molding a powder or granules containing the active compound, optionally with one or more accessory ingredients. Compressed tablets may be prepared by compressing, in a suitable machine, the compound in a free-flowing form, such as a powder or granules optionally mixed with a binder, lubricant, inert diluent, and/or surface active/dispersing agent(s). Molded tablets may be made by molding, in a suitable machine, the powdered compound moistened with an inert liquid binder.

The present invention is explained in greater detail in the following nonlimiting Examples. The examples which follow are set forth to illustrate the present invention, and are not to be construed as limiting thereof. As used herein, β arr2-GFP= β arrestin2 green fluorescent protein; GFP=green fluorescent protein; GPCR=G protein-coupled receptor; β ARK=beta adrenergic receptor kinase; GRK=G protein-coupled

receptor kinase; $\beta 2AR = \beta 2$ adrenergic receptor; HEK-293=human embryonic kidney cells; DMEM=Dulbecco's modified Eagle medium; and MEM=Minimal Essential Medium.

EXAMPLE 1

Materials and Methods

Materials: Isoproterenol was obtained from Sigma RBI. Anti-mouse antibody was obtained from Sigma Chemicals or Molecular Probes. Mouse monoclonal antibody against the 12CA5 epitope was obtained from Boehringer Mannheim. Cell culture media was obtained from Mediatech and fetal bovine serum from Atlanta Biologicals. Physiological buffers were from Gibco-Life Technologies Inc. Restriction enzymes were obtained from Promega or New England Biolabs, T4 ligase was from Promega, and Hot Tub DNA polymerase from Amersham. Commercially available plasmids containing variants of Green Fluorescent Protein were obtained from Clontech.

Cell Culture and Transfection: HEK-293 and COS cells were maintained and transfected as described by Barak et al., *Mol. Pharm.* 51:177 (1997). Cells containing both $\beta 2$ adrenergic receptor and β arrestin constructs were transfected with between 5-10 μ g of receptor cDNA in pcDNA1/AMP and 0.5-1 μ g of β arr2-GFP cDNA per 100 mm dish. GRKs were expressed using 5 μ g of transfected cDNA in pcDNA1/AMP per dish.

Confocal Microscopy: HEK-293 cells transfected as described above were plated onto 35 mm dishes containing a centered, 1 cm well formed from a hole in the plastic sealed by a glass coverslip. Primary and secondary antibody labeling of live cells were performed at 37.degree. C. for 30 minutes in media without serum in a 5% CO₂ incubator. Cells were washed three times between applications. Cells plated as above in MEM or DMEM buffered with 20 mM Hepes were viewed on a Zeiss laser scanning confocal microscope.

Sequestration: Flow cytometry analysis was performed using techniques known in the art, as described in Barak et al., *J. Biol. Chem.* 269:2790 (1994).

EXAMPLE 2

Construction of β arrestin2-GFP Plasmid

β arrestin2 cDNA in the plasmid pCMV5 was used as a template. Oligonucleotide primers surrounding a distal XhoI restriction site and the C-terminal stop codon of β arrestin2 were used to replace the stop codon with an in frame BamHI restriction site by directed mutagenesis (Valette et al. *Nucleic Acids Res.* 17:723 (1989); Attramadal et al., *J. Biol. Chem.* 267:17882 (1992); Lohse et al., *Science* 248:1547 (1990)). The XhoI, BamHI segment was isolated. This segment was ligated to the N-terminal portion of β arrestin cDNA (cut from pCMV5 by SacI and XhoI) in the polylinker of a plasmid that had been previously digested with SacI and BamHI and that contained S65T-Green Fluorescent Protein distal and in frame to the site of β arrestin cDNA insertion. Lohse et al., *Science* 248:1547 (1990). The resulting β arrestin-GFP construct was isolated following insertion and growth in *E. coli*. Constructs were verified by sequencing.

A linear model of the β arrestin2/S65T-GFP conjugate is provided in FIG. 1.

EXAMPLE 3

Characterization of β arr2-GFP Expressed by HEK-293 Cells

Homogenates of HEK-293 cells transformed with the plasmid of Example 2 were studied using known Western Blot techniques. The results showed that HEK-293 cells expressed both endogenous β arrestin and the β arr2-GFP conjugate.

Western Blots of homogenates of HEK-293 cells transfected with the plasmid of Example 2 and expressing β arr2-GFP were performed. An equal amount of homogenate material was loaded into each of two lanes (FIG. 2A). The left lane was exposed to anti- β arrestin antibody (Menard et al., *Mol. Pharm.* 51:800 (1997)), whereas the right lane was exposed to a mouse monoclonal antibody against GFP. The β arr2-GFP fusion

protein is approximately 50% larger than β arrestin2, and would thus be expected to migrate more slowly than β arrestin on SDS-Page.

Exposure to anti- β arrestin antibody revealed multiple bars (left lane); exposure to anti-GFP monoclonal antibody revealed a single bar (right lane). The position of endogenous cellular β arrestin2 is indicated by the intermediate bar in the left lane (β arr2). The heavy band just below 71,000 on the left lane (β arr2-GFP) is mirrored by a similar band in the right lane. In contrast, no band corresponding to endogenous cellular β arrestin 2 is observed with anti-GFP antibody exposure. The treatment of the right lane with anti-GFP antibody demonstrated that the slower band labeled by anti- β arrestin antibody contained GFP.

EXAMPLE 4

Biological Activity of β arrestin-GFP Conjugate

β arrestin activity can indirectly be assessed by measuring its effect on receptor sequestration (see Menard et al., *Mol. Pharm.* 51:800 (1997); Ferguson et al., *Science* 271:363 (1996)). The β 2AR normally sequesters poorly in COS cells, and this has been correlated to the relatively poor expression of endogenous β arrestins (see Menard et al. *Mol. Pharmacol.* 51:800 (1997); Ferguson et al, *Science* 271:363 (1996)).

Overexpression of exogenous β arrestin enhances β 2AR sequestration in these cells. To demonstrate that the β arr2-GFP conjugate is a biologically active β arrestin, COS cells overexpressing β arr2-GFP were examined for augmentation of β 2AR internalization, compared to the augmentation of β AR2 seen with the overexpression of β arrestin2. Results are shown in FIG. 2B.

Using epitope tagged β AR2 receptors, sequestration of β AR2 was studied in COS cells overexpressing either (1) exogenous β arrestin2 or (2) the β arr2-GFP conjugate. FIG. 2B shows the sequestration of β 2AR in COS cells with and without overexpressed β arrestin2 (left two bars) and with and without overexpressed β arr2-GFP (right two bars). Agonist mediated β 2AR sequestration increased from 15. \pm .7% to 39. \pm .5% in the

presence of overexpressed β arrestin2; overexpression of β arr2-GFP similarly increased agonist mediated β 2AR sequestration from 25. \pm .4% to 58. \pm .1%. Wild type β arrestin2 and β arr2-GFP enhanced β 2AR sequestration equally well above control levels, producing a 2.5 and 2.4 fold increase in β 2AR sequestration, respectively.

5 The above results indicated that the β arr2-GFP conjugate acts as a biologically active arrestin.

EXAMPLE 5

Agonist Mediated Translocation of β arr2-GFP

10 Agonist mediated translocation of the β arr2-GFP chimera from cell cytosol to membrane was studied using HEK-293 and COS cells transfected with plasmids containing cDNA for the β 2AR receptor and for the β arr2-GFP conjugate.

15 HEK-293 and COS cells were transfected with plasmids containing 10 μ g of cDNA for β 2AR and 0.5-1.0 μ g for β arr2-GFP. Cells were assessed using confocal microscopy to detect the inherent intracellular fluorescence of GFP.

20 Transfected HEK-293 cells are shown in FIG. 3A, where panel 1 depicts cells prior to the addition of β AR2 agonist, and panel 2 depicts cells following the addition of agonist. Transfected COS cells are shown in FIG. 3B, where panel 1 depicts cells just prior to the addition of β AR2 agonist, and panel 2 depicts cells ten minutes after the addition of agonist.

25 As shown in FIG. 3A, β arr2-GFP distribution in HEK-293 cells was initially cytosolic (panel 1). No significant nuclear or membrane enhancement was apparent. Following the addition of the β AR2 agonist isoproterenol to the cell medium, the real-time agonist-mediated redistribution of β arr2-GFP was viewed using confocal microscopy. Ten minutes after isoproterenol addition (saturating concentrations), enhancement of membrane fluorescence was seen with a concomitant loss of cytosolic fluorescence, indicating that the β arr2-GFP distribution had shifted to the membrane (panel 2). These results establish that in HEK-293 cells containing the β 2AR, β arr2-GFP

expressed by the cell is translocated from cytosol to membrane following the addition of a β AR2 agonist. Exposure of the test cells to GPCR agonist enhanced membrane bound fluorescence ten-fold over that seen prior to agonist exposure.

As shown in FIG. 3B, β arr2-GFP distribution in COS cells was initially cytosolic (panel 1). No significant nuclear or membrane enhancement was apparent. Following the addition of the β AR2 agonist isoproterenol to the cell medium, the real-time agonist-mediated redistribution of β arr2-GFP was viewed using confocal microscopy. Ten minutes after isoproterenol addition (saturating concentrations), enhancement of membrane fluorescence was seen with a concomitant loss of cytosolic fluorescence, indicating that the β arr2-GFP distribution had shifted to the membrane (panel 2). These results establish that in COS cells containing the β 2AR, β arr2-GFP expressed by the cell is translocated from cytosol to membrane following the addition of a β AR2 agonist.

Comparing FIGS. 3A and 3B shows that the fluorescent signal is reduced in COS cells as compared to HEK cells, reflecting the lower efficiency of sequestration of the β 2AR in COS cells. However, even in COS cells the shift of β arr2-GFP in COS cells from cytosol to membrane following the addition of β AR2 agonist is clearly discernible due to the fluorescence of the GFP moiety.

The above experiments with COS and HEK-239 cells were reproduced except that the β AR2 antagonist propranolol was added to the cell medium. Using confocal microscopy to visually track β arr2-GFP in the cell in real time, as above, indicated that no shift in β arr2-GFP from cytosol to membrane occurred in response to a β AR2 antagonist. As shown in FIG. 6E, addition of an agonist (middle panel) resulted in translocation of β arr2-GFP from cytosol to membrane; subsequent addition of an antagonist (right panel) reversed the translocation (compare to control, left panel).

Biochemical evidence indicates that β arrestins are predominantly cytosolic proteins. Ferguson et al, *Can. J. Physiol. Pharmacol.* 74:1095 (1996). The present results confirm that β arr2-GFP is distributed throughout the cytosol and excluded from the nucleus. These data also establish that β arr2-GFP is not

predominantly compartmentalized at the plasma membrane in the absence of agonist, but that upon exposure to an agonist the cellular β arr2-GFP shifts to the membrane. The present results further indicate that the shift of the β arr2-GFP conjugate in response to the addition of a G protein coupled receptor agonist can be detected optically as an enhancement of membrane fluorescence and/or a concomitant loss of cytosolic fluorescence, and that this response is rapidly observed.

EXAMPLE 6

Intracellular β arr2-GFP Targets Membrane Receptors

FIG. 4 shows the time course of β arr2-GFP redistribution to plasma membrane 12CA5(HA) tagged β 2AR in HEK-293 cells, as shown by confocal microscopy. The present example demonstrates that β 2ARs are the target of intracellular β arr2-GFP conjugate proteins. HEK-239 cells containing 12CA5(HA) tagged β 2AR receptors were studied. The receptors in the HEK-293 cells were reorganized into plasma membrane clusters (Row A) by crosslinking with a mouse monoclonal antibody directed against an N-terminal epitope, followed by Texas Red conjugated goat anti-mouse antibody. In FIG. 4, the three panels of Row A show the same HEK-293 cell with β AR2 receptors reorganized into plasma membrane clusters.

HEK-239 cells were then exposed to agonist (isoproterenol added to cell medium, as above); the three panels of Row B in FIG. 4 were taken consecutively after agonist addition (left to right, at 0, 3 and 10 minutes post agonist addition). The real-time redistribution of β arr2-GFP to the receptors over a ten minute time period is thus demonstrated by comparing the panels of Row A and Row B of FIG. 4. In FIG. 4, arrows indicate areas of colocalization and the bar=10 microns.

FIG. 4 demonstrates that the geometry of the agonist-induced time dependent translocation of β arr2-GFP to the plasma membrane mimicked the distribution of pre-aggregated β 2ARs. This indicates that the primary site targeted by β arrestin is the β 2AR or a closely associated component.

EXAMPLE 7

Intracellular β arr2-GFP Targets Membrane Receptors

It has been postulated that phosphorylation of GPCRs by GRKs facilitates desensitization by increasing their affinity for β arrestins. Gurevich et al., *J. Biol. Chem.* 268:16879 (1993); Gurevich et al., *J. Biol. Chem.* 268:11628-11638 (1993); Ferguson et al., *Can. J. Physiol. Pharmacol.* 74:1095 (1996). When expressed in HEK-293 cells and exposed to agonist, mutant Y326A- β 2ARs are not significantly phosphorylated by endogenous GRKs. Barak et al., *Biochem.* 34:15407 (1995); Ferguson et al., *J. Biol. Chem.* 270:24782 (1995). This phosphorylation impairment in Y326A- β AR2s is reversed by overexpression of GRKs in the same cell. Menard et al., *Biochem.* 35:4155 (1996). The Y326A mutant receptor was used to investigate β arrestin affinity in vivo; the effect of overexpressed GRK on the Y326A-B2AR interaction with β arr2-GFP was shown.

Y326A- β 2AR and β arr2-GFP were co-transfected into HEK-239 cells, in the absence and presence of co-transfected GRK. If phosphorylation of GPCRs by GRKs facilitates desensitization by increasing their affinity for β arrestins, then overexpression of GRK would result in a noticeable difference in β arr2-GFP translocation.

FIG. 5 shows the influence of overexpressed GRK on the redistribution of β arr2-GFP in HEK-293 cells expressing the Y326A phosphorylation impaired β 2AR. Cells without (Row A) and with (Row B) overexpressed GRKs were exposed to agonist, and the real-time redistribution of β arr2-GFP was observed. Without added GRK, β arr2-GFP translocation in response to agonist proceeded poorly, as shown in Row A of FIG. 5. β arr2-GFP translocation in cells containing overexpressed GRK (Row B) was more robust, indicating an increased affinity of β arr2-GFP for receptor and the relationship of phosphorylation and β arrestin activity.

EXAMPLE 8

Testing of Additional Receptors in the β 2AR/rhodopsin Subfamily

Twelve different members of the β 2AR/rhodopsin subfamily of GPCRs have been studied. Cells expressing a particular GPCR, and containing β arrestin-GFP chimeric proteins were exposed to known agonists for the GPCR being studied. In each case, an observable translocation of the β arrestin-GFP chimeric proteins from the cell cytosol to the cell membrane was produced within minutes following addition of the GPCR agonist (data not shown).

EXAMPLE 9

Production of β Arrestin Knockout Mice

Because GPCRs, such as the substance P receptor and the opioid receptors, participate in processing the sensation of pain, we characterized analgesic responses through the μ opioid receptor (μ OR) in mice lacking β arrestin-2. In the clinical setting, morphine is currently the most effective drug for alleviating intense and chronic pain. The antinociceptive (blocking of pain perception) actions of morphine are mediated through stimulation of the μ OR, as demonstrated by the lack of morphine analgesia observed in knock out mice deficient in the μ OR (H. Matthes et al., *Nature* 383, 819 (1996). B. Kieffer, *Trends Pharmacol Sci* 20, 19 (1999); 1. Sora et al., *Proc Natl Acad Sci USA* 94, 1544 (1997)). Nevertheless, the neuronal signaling mechanisms mediating analgesia through μ ORs and morphine remain poorly understood. Moreover, the contribution of GPCR desensitization to the onset and duration of analgesia has been unclear.

β arrestin-2 knockout (β arr2-KO) mice were generated by inactivation of the gene by homologous recombination. A bacteriophage λ library of mouse 129SvJ genomic DNA (Stratagene, La Jolla, CA) was screened with the rat β arr2 cDNA (H. Attramadal et al., *J Biol. Chem.* 267, 17882 (1992)). Positive phages were identified and analyzed by restriction digest. A 12-kb β arr2 fragment was digested with Bam HI, subcloned into

pBluescript KS(-) and sequenced. The targeting vector was assembled by blunt-end ligation of a pHSV-TK cassette (from pIC19R/MCI-TK, M. R. Capecchi, University of Utah), a 2.8-kb Nco I-Bam HI β arr2 fragment, a pGK-neo cassette (from plasmid pD383, R. Hen, Columbia University) which replaced the 0.8kb Bam HI-Hind III fragment of β arr2, and a 4.5 kb Hind III β arr2 fragment into pBluescript KS(-). This targeting vector was linearized with Not I and was electroporated into mouse embryonic stem cells. Genomic DNA from transfectants resistant to G418 and gancyclovir were isolated and screened by Southern (DNA) blot analysis using a 0.2 kb 5' external β arr2 probe and a 0.3 kb 3' external β arr2 probe. Chimeric animals were generated by microinjecting these ES cells into C57BL/6 blastocysts. Five chimeric male pups were obtained and mated with C57BL/6 females. Germline transmission was confirmed by Southern blotting. Heterozygous, offspring were intercrossed to obtain homozygous mice. Wild-type and mutant mice used in this study were age-matched, 3 to 5 month old, male siblings. For protein immunoblot analysis, whole cell lysates were prepared by polytron homogenization in lysing buffer [10 mM Tris (pH 7.4), 5 mM EDTA, 1 protease inhibitor tablet/ 10 mL (Roche Molecular Biochemicals, Indianapolis, Indiana, USA), 1% nonidet-40]. Polyacrylamide gels were loaded with 25 μ g protein/ lane and equivalent protein loading was confirmed by Ponceau S staining of the gels. After transfer to polyvinylidene difluoride (PVDF) membranes, proteins were blotted with polyclonal antibodies to β arrestin-2 or β arrestin-1 [H. Attramadal et al., *J Biol.Chem.* 267, 17882 (1992)]. Bands were visualized with secondary antibody conjugated to horseradish peroxidase and an enhanced chemiluminescence detection system (Amersham, Piscataway, NJ). All experiments were conducted in accordance with the NIH guidelines for the care and use of animals.

EXAMPLE 10

Identification of β Arrestin Knockout Mice

Mice lacking β arrestin-2 were identified by Southern DNA blot analysis (Fig. 7A)

and the absence of β arrestin-2 was confirmed by protein immunoblotting of extracts from brainstem, periaqueductal. gray (PAG) tissue, spleen, lung and skin (Fig. 7B). Wild-type, heterozygous, and homozygous mutant mice had similar amounts of β arrestin-1 in the brain regions examined (Fig. 7B), arguing against compensatory upregulation of β arrestin-1 in the absence of β arrestin-2. The β arr2-KO mice were viable and had no gross phenotypic abnormalities. However, after administration of morphine, obvious differences became apparent between the genotypes.

EXAMPLE 11

Evaluation of Morphine-Induced Antinociception in β arrestin Knockout Mice

Morphine-induced antinociception was evaluated by measuring response latencies in the hot plate test. We used a dose of morphine (10 mg/kg) and route of administration (s.c.) well established to induce analgesia in many strains of mice (F. Porreca et al., *J Pharmacol Exp Ther* **230**, 341 (1984). J. Belknap et al., *Physiol Behav* **46**, 69 (1989). A Gardmark et al., *Pharmacol Toxicol* **83**, 252 (1998). G. Elmer et al., *Pain* **75**, 129 (1998)). The analgesic effect of morphine was significantly potentiated and prolonged in the knockout mice as compared to that in their wild-type littermates (Fig.8). Such robust responses to morphine were not only absent in the wild-type littermates (Fig. 8) but also in the parental mouse strains (C57BL/6 and 129SvJ) used to generate this knockout. Four hours after the morphine injection, β arr2-KO mice still exhibited significant analgesia (% maximum possible effect = $31 \pm 0.4\%$); whereas, in control wild-type littermates, the analgesic effects of the same dose of morphine waned after about 90 minutes. β arr2 +/- mice were nearly as responsive to morphine as the β arr2-KO mice; however, this may reflect the imposed limit of the hot plate assay (30 seconds), which is designed to prevent prolonged exposure of the mice to pain. Basal responses to the hot plate did not differ between genotypes (wild type: 6.2 ± 0.3 sec., n=25; β arr2-KO: 6.1 ± 0.4 sec., n=27). The differences in morphine-induced analgesia between the genotypes are unlikely to be due to pharmacokinetic differences in morphine metabolism, because the concentrations of

morphine in blood, as determined by mass spectroscopy analysis, did not differ between wild type and β arr2-KO mice 2 hours after morphine injection (Mice were injected with morphine (10 mg/kg, subcutaneous). After 30 minutes or 2 hours, wild-type mice were killed and blood was collected in vials containing sodium fluoride and potassium-oxalate. Morphine concentration in blood samples pooled from 3 mice per sample were 1,500 ng/mL after 30 min., and 83 ng/mL blood after 2 hours as measured by mass spectroscopy analysis [Occupational Testing Division of LabCorp, Inc., Research Triangle Park, North Carolina, USA]. In similar experiments, β arr2-KO mice had a concentration of 93 ng/mL in the blood after 2 hours.

EXAMPLE 12

Evaluation of Low Dosage Morphine in β arrestin Knockout Mice

Lower doses of morphine were also tested in these assays. Even at doses of morphine (1 mg/kg, s.c.) that were sub-analgesic in wild type mice, β arr2-KO animals displayed a significant increase in their nociceptive thresholds (Fig. 9). At 30 minute intervals, immediately following the antinociception test, mice were given repeated cumulative doses of morphine resulting in final concentrations of 5, and 10 mg/kg (I. Sora et al., *Proc Natl Acad Sci USA* **94**, 1544 (1997)). At the highest cumulative dose, mice reached similar levels of antinociception as seen in Fig. 8, in which this amount of morphine was administered in a single injection. At every dose, the β arr2 KO animals experienced greater antinociception after morphine treatment than did their wild-type littermates.

EXAMPLE 13

Evaluation of Morphine Antagonists in β arrestin Knockout Mice

To test whether the analgesic effects of morphine were mediated by actions at the μ OR, mice were treated with various antagonists. Naloxone (2.5 mg/kg, subcutaneous injection) which immediately reverses the effects of opiates, was given 30 minutes after

EXAMPLE 15

Radioligand Binding Assays

To investigate whether the μ OR population was altered in the KO mice, radioligand binding analysis on membranes prepared from different brain regions was performed. Brain regions were dissected and immediately frozen in liquid nitrogen and were stored at -80°C for less than 1 week before use. Samples were placed on ice and homogenized by polytron in membrane preparation buffer [50 mM Tris (pH 7.4), 1 mM EDTA, 3 mM MgCl_2] and crude membranes were prepared by centrifugation at 20,000xg for 15 min at 4°C . Membranes were resuspended in either 50 mM Tris-HCl (pH 7.4) for radioligand binding assays or in assay buffer [50 mM Tris-HCl (pH 7.4), 100mM NaCl, 3 mM MgCl_2 , 0.2mM EDTA] containing 10 μ M GDP for [^{35}S] GTP γ S binding assays. For both binding assays, reactions were terminated by rapid filtration over GF/B filters (Brandel, Inc., Gaithersburg, MD) using a Brandel cell harvester (Brandel, Inc., Gaithersburg, MD). Filters were washed 3 times with ice cold 10 mM Tris-HCl (pH 7.4) and then counted in a liquid scintillation counter. Hypothalamus, brain stem, and periaqueductal gray (PAG) regions were chosen because they contain μ ORs and are implicated in the regulation of pain and body temperature (D. Mayer and D. Price, *Pain* **2**, 379 (1976). T. Yaksh et al., *Prog Brain Res* **77**, 371 (1988). D. J. Smith., et al., *Eur J Pharmacol* **156**, 47 (1988)). Data are given in Table I. Saturation binding studies with ^3H -naloxone, at concentrations that preferentially label the μ OR, revealed a single high affinity binding site, which represents the μ OR. The number and affinity of μ ORs did not significantly differ between the two genotypes in any of the brain regions examined.

Table I
H-Naloxone binding in brain regions of Wild Type and Knockout mice.¹

Brain region	Wild Type		β arr2-Knockout	
	B_{MAX} (fmol/mg)	K_D (nM)	B_{MAX} (fmol/mg)	K_D (nM)
PAG	132±9	4.0±0.1	144±13	3.0±0.8
Brainstem	49±7	1.5±0.2	54±9	3.0±0.8
Hypothalamus	103±18	6.2±1.6	89±8	3.8±0.2

¹Saturation binding assays were performed on membranes from different brain regions (50-100 μ g/tube) with increasing concentrations of ³H-naloxone (0-12 nM, 52.5 Ci/mmol, Amersham, Piscataway, New Jersey, USA). Nonspecific binding was determined in the presence of 10 μ M naloxone. Membranes were incubated at 25°C for 1 hour. Binding parameters were determined via Scatchard analysis of specific binding. Data are the mean \pm S.E.M. of 3-4 experiments performed in duplicate.

Additional evidence for increased sensitivity of the μ OR in β arr2-KO animals was obtained in biochemical experiments. We measured agonist-stimulated binding of [³⁵S]GTP γ S to G proteins in isolated membranes the most proximal manifestation of GPCR activation (D. Selley et al., *Mol Pharmacol* **51**, 87 (1997)). Because morphine acts *in vitro* to stimulate μ , δ , and κ opioid receptors, the μ OR-selective agonist, [*D*-Ala², MePhe⁴, Gly⁵-*ol*]enkephalin (DAMGO), was used to specifically activate G protein coupling to μ ORs. DAMGO stimulated more [³⁵S]GTP γ S binding in membranes derived from β arr2-KO mice than in those derived from wild-type littermates (Fig. 11). Similar results were also obtained in brainstem membranes (data not shown). The amount of G α proteins (G_{i/o/z}) as determined by protein immunoblotting, did not vary between the genotypes (data not shown). These observations suggest that there is enhanced coupling of μ ORs to G proteins in tissues derived from β arr2-KO mice. Although the enhanced analgesia induced by morphine may involve complex neurological- signaling, this biochemical evidence supports the interpretation that the enhanced physiological

responsiveness in the knockout animals results from increased sensitivity of signaling by the μ OR.

These studies demonstrate in an animal model that the absence of β arrestin-2 can affect the efficacy of GPCR activation. In transfected cultured cells, the degree of β_2 -adrenergic receptor signaling is dependent upon the cellular complement of GRK2 and GRK3 and β arrestins (L. Menard et al., *Mol Pharmacol* **51**, 800 (1997); S. Mundell et al., *Biochemistry* **38**, 8723 (1999)). These observations, along with those presented here, directly support the proposed role of β arrestin-2 in preventing further receptor-G protein coupling and mediating desensitization of the GPCR. Moreover, β arrestins are not only involved in the dampening of GPCR responsiveness after agonist stimulation, but also influence the sensitivity of the response.

The simplest interpretation of these results is that μ OR signaling is regulated by β arrestin-2. However, in transfected cells, morphine fails to induce the internalization of the μ OR and a GFP-tagged β arrestin-2 fails to translocate to μ OR overexpressed in cell culture upon exposure to morphine (J. Arden et al., *J Neurochem* **65**, 1636 (1995). D. Keith et al., *J Biol Chem* **271**, 19021 (1996); J. Whistler and M. von Zastrow, *Proc Natl Acad Sci USA* **95**, 9914 (1998); J. Zhang et al., *Proc Natl Acad Sci USA* **95**, 7157 (1998)). Interestingly, these *in vitro* studies have been conducted with the rat μ OR or the mouse MOR1 which are not particularly rich in potential phosphorylation sites. Several splice variants of the μ OR are present in mouse brain that contain several potential phosphorylation sites (Y. Pan et al., *Mol Pharmacol* **56**, 396 (1999)). Some of these isoforms can contribute to morphine-induced analgesia. The involvement of these receptors might explain the differences between the *in vitro* studies and those with the β arr2-KO mice.

The β arr2-KO mice were very similar in phenotype to their wild type littermates and other GPCR-directed drugs did not necessarily elicit different responses between the genotypes. For example, locomotor responses to dopamine receptor stimulation by cocaine and apomorphine were not enhanced (data not shown). These observations

suggest that various GPCRs are differentially affected by the loss of β arrestin-2. Other regulatory elements, such as GRKs or β arrestin-1, could compensate for the lack of β arrestin-2, or the receptors could vary in their requirement for β arrestin interaction for their regulation.

- 5 The foregoing is illustrative of the present invention, and is not to be construed as limiting thereof. The invention is defined by the following claims, with equivalents of the claims to be included therein.